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Polarization of the Golgi apparatus and the microtubule-organizing center within cloned natural killer cells bound to their targets

(cytotoxicity/directed secretion/double immunofluorescence)

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ABSTRACT **In cell couples formed between a cloned murine natural killer (NK) cell and either of two target cells, we have obtained evidence by immunofluorescence observations for a rapid coordinate repositioning of two organelles, the microtubule organizing center and the perinuclear Golgi apparatus, inside the NK cell to face the region of contact with the target cell. With microtubule-disrupting drugs, which also cause a dispersion of the Golgi apparatus throughout the periphery of the NK cell, it was found that target cell lysis is reversibly inhibited. It is proposed that the coordinate repositioning of the two organelles serves the function of directing secretory vesicles derived from the Golgi apparatus to the bound target cells, the secretory vesicles containing components that induce target cell lysis.**

Natural killer (NK) cells are thought to provide an important primary defense mechanism against microbial infections and the development of tumors. *In vitro*, NK-containing lymphoid cell populations have been shown to induce the lysis of a number of types of neoplastic target cells (TC), but the detailed mechanisms of cell killing have not been readily accessible to study because of the poorly defined character and low frequency of NK cells in lymphoid populations. Recently, however, we have been able to clone and establish murine NK cells as permanent *in vitro* lines (1). Such NK clones provide an important means to explore the molecular basis of their cytotoxic activity. Electron microscopic studies have indicated (2), for example, that upon binding of cloned NK cells to their TC, the NK cell secretes multibilayer and specific tubular structures that appear to collect in the TC plasma membrane, and it was proposed that one or more of these secretory products was responsible for TC lysis. Significant evidence that NK cell secretion may be important in the cytolytic mechanism had also been obtained by others (cf. ref. 3).

At this point we were struck by the possible relevance to NK cytolytic mechanisms of studies that were being carried out with an entirely different phenomenon, the directed migration of cultured fibroblasts. It was observed (4) that when fibroblasts were stimulated to move in a particular direction, the Golgi apparatus (GA) and the microtubule-organizing center (MTOC) were coordinately and rapidly repositioned forward of the nucleus in the direction of subsequent cell migration. The GA is the organelle in which components destined to be inserted into the plasma membrane of the cell, and components destined to be secreted from the cell, are processed and packaged (5). The MTOC is the region of the interphase cell, including the centriole pair, from which the cytoplasmic microtubules emanate (6). From these results we had suggested (4) that the purpose of the GA/MTOC reorientation in the fibroblasts was to achieve a cytoskeletally directed vectorial traffic of GA-derived vesicles to the leading edge of the cell, to insert new membrane mass into the leading edge (7–9) and to direct secretion there as well.

If directed secretion from the NK cell to the TC was involved in cell killing, it seemed possible that a similar coordinate repositioning of the GA and MTOC might occur inside a NK cell towards its bound TC. This has now been investigated, as in our previous study (4), by double indirect immunofluorescence microscopy, using rabbit antibodies specific for a membrane component of the GA (10) and guinea pig antibodies to tubulin to label the MTOC. We have obtained evidence that, indeed, shortly after the cloned NK cells bound to their targets, a rapid coordinate GA/MTOC repositioning occurred in the NK cells (but not in TC) facing the area of cell–cell contact. Furthermore, when the NK cell microtubules were depolymerized by nocodazole, and an MTOC subsequently formed in the presence of a mixture of nocodazole and taxol (11), the orientation of the MTOC inside the NK cells that were bound to TC was random. Under these conditions, TC lysis was strongly inhibited. When the drugs were subsequently washed out of the NK–TC couples, resulting in the reassembly of intracellular microtubules in the NK cells, the MTOC became reoriented to face the bound TC. Under these conditions TC lysis was largely recovered. These results are consistent with the proposal that, upon binding of a NK cell to a sensitive target, a rapid reorientation of the GA/MTOC occurs inside the NK cell that serves the function of directing secretory vesicles derived from the GA to the bound TC.

MATERIALS AND METHODS

Cell Lines. Cloned NK cells derived from C57BL/6 mice, isolated as previously described for BALB/c NK cells (1), were employed. In all experiments clone NK B6 1A2 (12), maintained in medium conditioned by concanavalin A-induced spleen cell proliferation, was used. Two TC sensitive to NK lysis were employed: the A-mouse thymic lymphoma line YAC-1 ($H\text{-}2^a$) and the BALB/c myeloma line S194 ($H\text{-}2^d$). Both lines were propagated *in vitro* either in RPMI 1640 medium supplemented with 10% fetal bovine serum (YAC-1) or in Dulbecco's modified Eagle's medium supplemented with 10% horse serum (S194).

Antibody and Other Reagents. Rabbit antibodies specific for membranes of the GA of rat cells were the gift of D. Louvard of the Institut Pasteur (10). Affinity-purified rabbit and guinea pig antibodies to chick brain tubulin were used as in our previous studies (4). A rat anti-Thy-1 monoclonal antibody, T24/31.7 (13), was used to identify NK cells. Rhodamine and fluorescein conjugates of affinity-purified and cross-absorbed goat

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Abbreviations: NK, natural killer; TC, target cell(s); GA, Golgi apparatus; MTOC, microtubule-organizing center(s); CTL, cytotoxic T lymphocytes.

antibodies to rabbit IgG and guinea pig IgG (4), and a fluorescein conjugate of an $F(ab')_2$ fragment of goat anti-rat IgG antibodies (Cappel Laboratories, Cochranville, PA), were employed as secondary immunolabeling reagents. Nocodazole (Aldrich, Milwaukee, WI) and taxol (courtesy of M. Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute) were used as supplied.

NK–TC Conjugation and Cytotoxicity Assay. NK B6 1A2 cells ($5–10 \times 10^5$) were mixed with an equal number of YAC-1 or S194 TC in 1 ml of RPMI 1640 medium supplemented with 5% fetal bovine serum. The mixture was centrifuged for 5 min at $100 \times g$, and the cells were then gently resuspended with a Pasteur pipet. Aliquots of $5–10 \times 10^4$ cells were plated on poly(L-lysine)-treated coverslips and placed for 10 min at 37°C in a CO_2 incubator. With S194 cells as targets (see below), the plated cells were then immunofluorescently labeled with the anti-Thy-1 antibody reagents and were subsequently fixed with 3% (wt/vol) formaldehyde and permeabilized by treatment with 0.15% Triton X-100 for 5 min. With YAC-1 targets, the plated cells were directly fixed and permeabilized as above.

For assays of cytolytic activity, TC were labeled with ^{51}Cr, washed in medium, and mixed with NK cells at various ratios of killer to target cells. ^{51}Cr release was measured and the percent cytotoxicity was calculated (1).

In the experiments in which microtubule assembly in the NK cells was altered, NK cells were treated with nocodazole (10 μg/ml) in RPMI 1640 medium supplemented with 10% fetal bovine serum for 90 min at 37°C (a treatment that depolymerizes all the cytoplasmic microtubules). The cells were then centrifuged down and resuspended in medium containing nocodazole at 10 μg/ml and 5 μM taxol. Under these conditions, microtubule assembly occurs only to a limited extent and is confined to the region around the centriole pair (11). For immunofluorescence studies of conjugates, the NK cells were then added to an equal number of TC and were allowed to interact for up to 35 min after mixing, before fixation as described above.

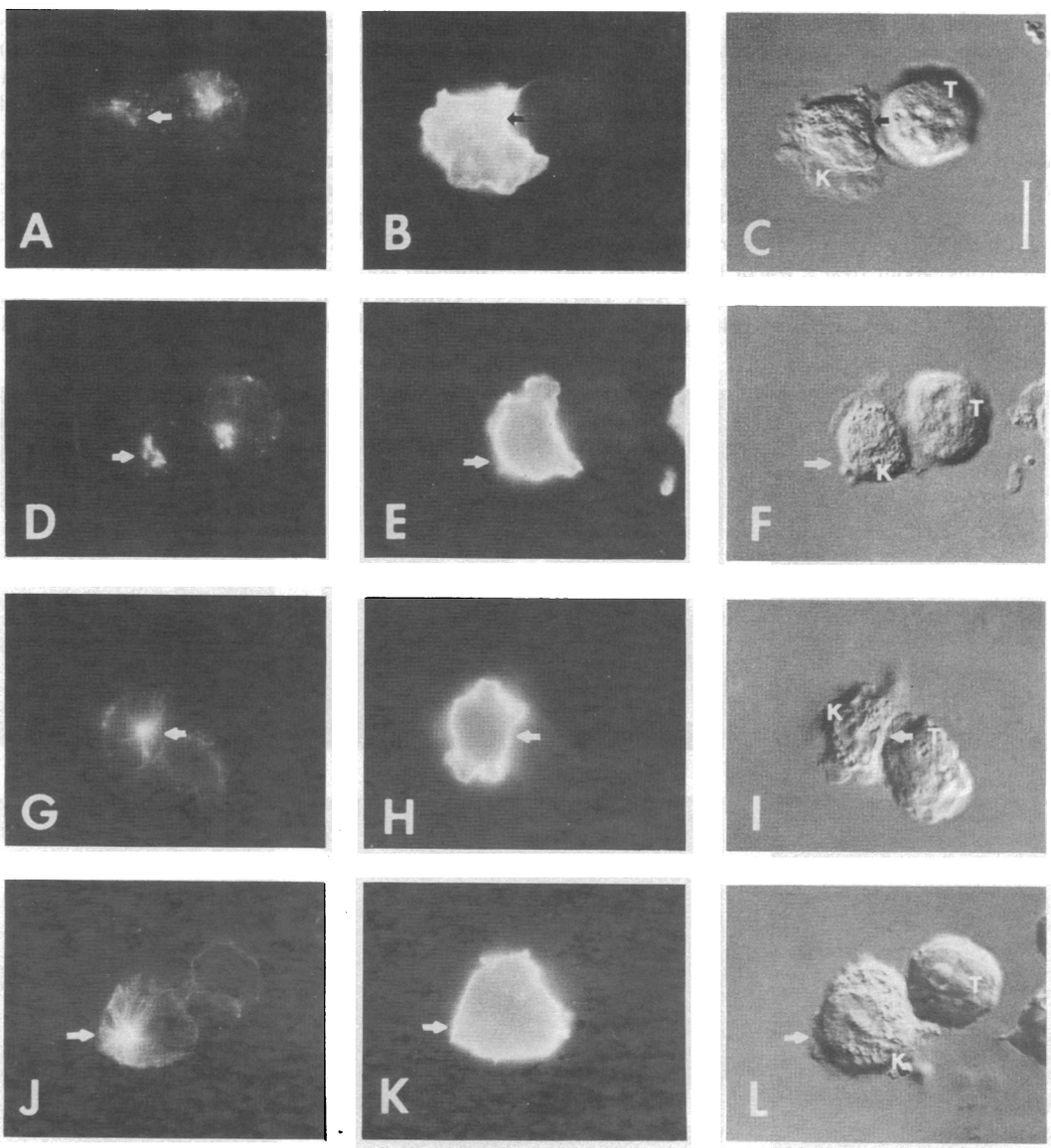

FIG. 1. Light microscopy of couples of cloned NK cells (K) and S194 TC (T), 20 min after mixing the cells. Nomarski optics images of four different couples are shown (*C, F, I,* and *L*). The NK cell in each couple is identified by immunofluorescence labeling with anti-Thy-1 antibodies (*B, E, H,* and *K*). The corresponding couples were also immunofluorescently labeled to reveal either their GA (*A* and *D*) or their MTOC (*G* and *J*). The couples in *A–C* and *G–I* are representative of the majority (70%) of couples in the population, those in *D–F* and *J–L*, the minority (30%). The arrow in *A* points to the site in the NK cell where the GA is facing towards its target cell, whereas in *D* it is facing away. In *G*, the arrow points to the site in the NK cell where the MTOC is facing towards the target cell, whereas in *J* it is facing away. The bar in *C* indicates 10 μm.

In some experiments, the microtubules in the NK cells were allowed to repolymerize by suspension of the NK–TC couples in drug-free medium for 15 min before fixation. For the cytolytic assays, the drug-treated NK cells were added to ^{51}Cr-labeled TC and ^{51}Cr release was monitored as above.

Light and Immunofluorescence Microscopy. The NK cells in NK–TC conjugates were recognized in the microscope as follows: (*i*) In experiments utilizing S194 cells as TC, the unfixed NK cells were first specifically immunofluorescently labeled for Thy-1 on their surfaces. (*ii*) When YAC-1 TC were used, the NK cells could be distinguished by their morphology and size in Nomarski optics. In the former case, the surface labeling for Thy-1 utilized the monoclonal rat anti-Thy-1 antibody followed by the fluoresceinated $F(ab')_2$ of goat anti-rat IgG, and then intracellular rhodamine immunolabeling for either the GA or the microtubules was carried out on the fixed and permeabilized cells. With YAC-1 TC, double immunofluorescence labeling for the GA and microtubules was performed on the cells. Only 1:1 NK–TC cell pairs were analyzed. The MTOC or GA was scored as facing the contact area with its partner cell if it was located between the nucleus and the contact area. In each experiment at least 100 pairs of NK–TC were examined.

RESULTS

Immunofluorescence Observations of NK–S194 Couples. Because the NK cells were labeled with an anti-Thy-1 antibody, the cells were further immunostained separately for localization of their GA or their MTOC. Already at the earliest time (about 20 min after cell mixing) about 70% ± 5% of the NK cells had their GA and their MTOC facing the TC (Fig. 1 *A–C, G–I*), whereas 30% ± 5% had their GA or MTOC oriented away from the TC (Fig. 1 *D–F, J–L*). By 30 min later, the GA and the MTOC in essentially all the NK cells were found facing the TC contact area. In contrast to the NK cells, no such correlation could be observed with respect to the intracellular location of the GA or MTOC inside the S194 targets.

Immunofluorescence Observations of NK–YAC-1 Couples. The NK cells and the YAC-1 cells could be distinguished by morphological differences, so the cells were double immunofluorescently stained for the GA and the MTOC. The immunolabeled GA and MTOC in any single cell were invariably localized close to one another (Fig. 2). At the earliest time about 65% ± 5% of the GA and MTOC in the NK cells were found to be facing the YAC-1 cells (Fig. 2 *A–C*), whereas 35% ± 5% of the GA and MTOC were facing away (Fig. 2 *D–F*). By 20 min later the GA and MTOC in essentially all of the NK cells were localized forward of the nucleus towards the contact site with the TC.

When the microtubules of the NK cells were depolymerized by using nocodazole followed by nocodazole/taxol, prior to the addition of YAC-1 cells, we found that binding of the NK and YAC-1 cells subsequently occurred. However, either 25 min after mixing (Fig. 3 *A* and *D*, *B* and *E*) or 35 min after mixing (not shown) there was no correlation between the intracellular location of the MTOC in the NK cells and the target-binding site. By 35 min with control NK–YAC-1 couples the MTOC in the NK cells were essentially all oriented facing the target cells. However, within 15 min after the drugs were washed away, the MTOC that were initially randomly oriented became oriented towards the TC in essentially all the NK cells that had reassembled microtubules (Fig. 3 *C* and *F*).

Cytotoxicity in Relation to Microtubule Integrity. Depolymerization of the microtubules by treatment with nocodazole

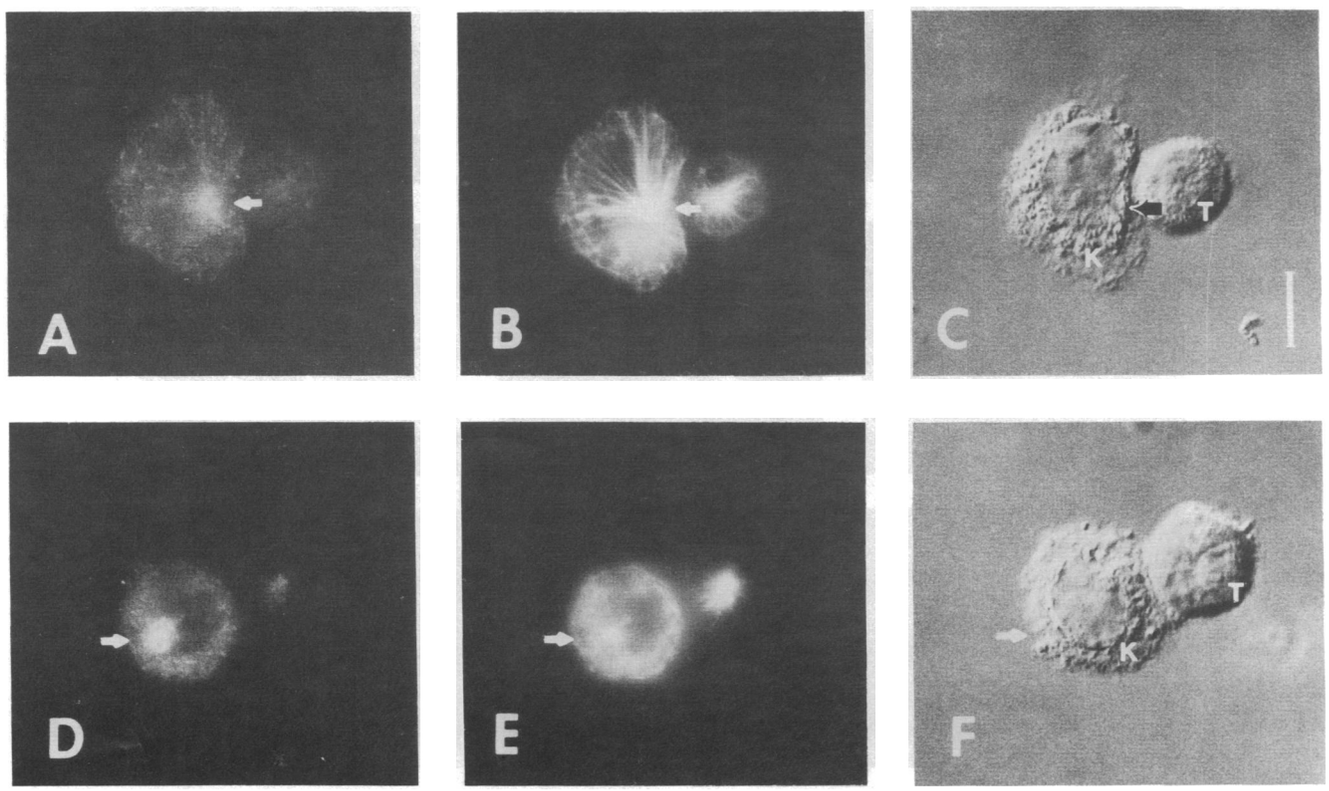

FIG. 2. Couples of NK cells (K) and YAC-1 TC (T), observed 15 min after mixing. Nomarski optics images of two different couples are shown (*C* and *F*). The corresponding cells were double immunofluorescently labeled to reveal their GA (*A* and *D*) and their MTOC (*B* and *E*). The example in *A–C* is representative of the majority (65%) of the population of couples, that in *D–F* of the minority (35%). The GA (arrow in *A*) and the MTOC (arrow in *B*) in the NK cell are coordinately positioned facing towards the TC, whereas in the example in *D–F*, the two organelles are coordinately positioned facing away from the target cell. Bar in *C* indicates 10 μm.

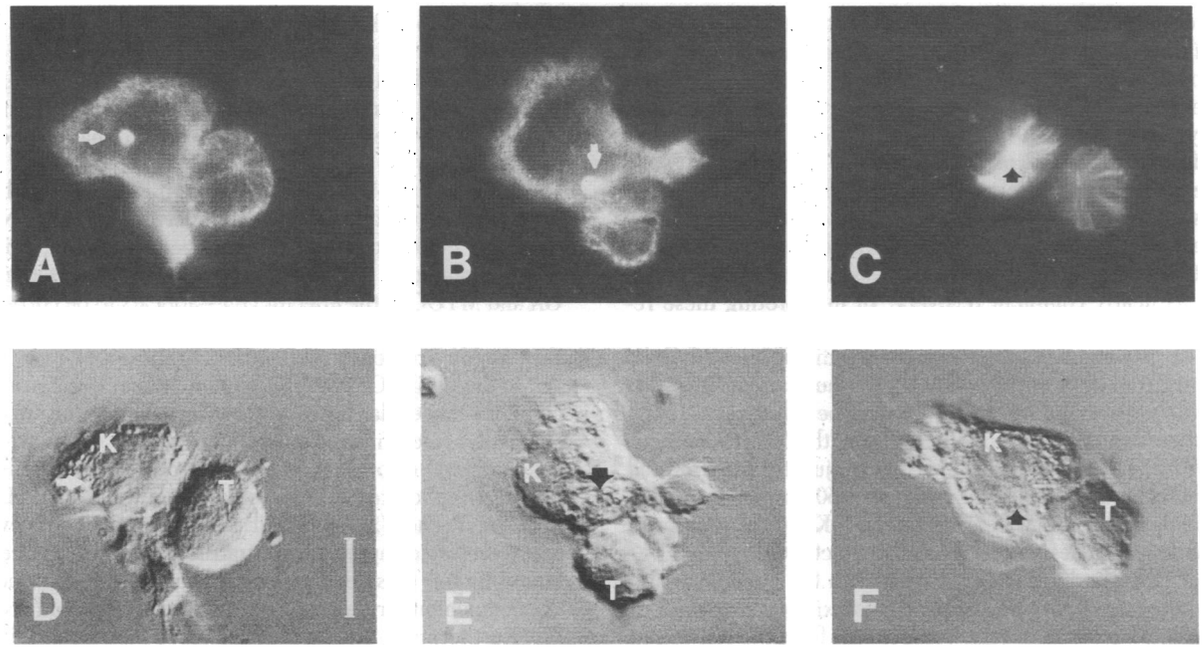

FIG. 3. Couples of NK cells (K) and YAC-1 TC (T), visualized by Nomarski optics (*D–F*). To prepare the cell couples shown in *A* and *D* and *B* and *E*, the cytoplasmic microtubules in the NK cells alone were depolymerized by nocodazole, followed by nocodazole/taxol. The cells were then bound to the targets, and 25 min later the couples were examined by immunofluorescence labeling of the residual MTOC (arrows in *A* and *B*). *A* and *D* and *B* and *E* are two examples of a population showing random orientations of the NK MTOC with respect to the bound target cells. By 15 min after washing out the drugs, the couple in *C* and *F* is representative of essentially the entire population, showing the MTOC (arrow in C) facing towards the bound target cell. Bar in *D* indicates 10 μm.

followed by nocodazole/taxol abolished the lytic activity of the NK cells towards YAC-1 targets (Fig. 4). It should be emphasized that this drug treatment did not impair the binding of the NK cells to their TC (Fig. 3). This inhibition of cytotoxicity appears to be related primarily to the action of nocodazole, since similar inhibition was observed when nocodazole alone (no taxol) was present throughout the experiment (not shown). Moreover, the cytolytic activity of the NK cells could be almost fully recovered when nocodazole was washed away (Fig. 4).

DISCUSSION

The objectives of these experiments were to explore the hypothesis that NK cell-mediated cytotoxicity involves a directed secretion of cytotoxic components from the NK cell to its bound target and the possibility that the orientation of the GA and the MTOC inside the NK cell is critical to such directed secretion. Our results indeed strongly suggest that the binding of a cloned

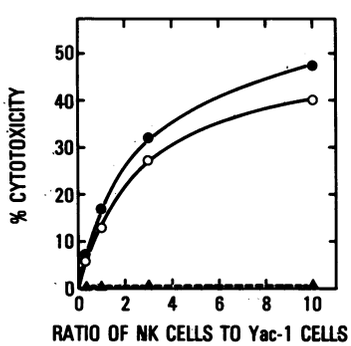

FIG. 4. Effects of microtubule assembly status on NK cytotoxicity. NK cells were incubated in normal medium (●), treated with nocodazole followed by nocodazole/taxol (▲), or treated with nocodazole followed by washing out of the drug with normal medium (○). The NK cells were then mixed with YAC-1 cells in the respective media and cytotoxicity was assayed after 4.5 hr.

NK cell to a sensitive target triggers an early sequence of events that leads to a rapid coordinate repositioning of the GA and MTOC inside the NK cell to face towards the contact area with the TC. This is our interpretation of the facts that at the earliest time we made observations (20 min after mixing the NK and TC) 65–70% of the NK cells that were bound to their targets exhibited a GA/MTOC orientation towards the cell–cell contact area; in the following 20–30 min, the GA/MTOC in the remaining 30–35% of the NK–TC pairs acquired such an orientation. An alternative possibility is that, after the initial NK–TC conjugation, the two cells crawled over one another until the cell contact area faced the GA/MTOC in the NK cell. However, time-lapse video observations of a large number of NK–YAC-1 conjugates (not shown) did not provide any evidence for such movements, and we therefore exclude this possibility.

We assume that, at the instant of NK–TC binding, the GA/MTOC orientation inside the NK cell was random with respect to the cell–cell contact area. The rate of the organelle repositioning in the NK cells that is indicated by these observations is similar to the rate of GA/MTOC reorientation we observed inside cultured fibroblasts that had received a stimulus to undergo directed migration (4). In that case, the reorientation had occurred in about half of the responding cells within 5 min after stimulation, and in about 70% of the cells within 20 min.

That NK–TC binding normally provides a signal for GA/MTOC reorientation in the NK cell is further indicated by the experiments involving microtubule assembly status in the NK cells. With NK cells whose cytoplasmic microtubules were depolymerized by nocodazole treatment followed by nocodazole/taxol, binding to TC cells left the MTOC in the NK cells randomly orientated with respect to the cell–cell contact area. When the cytoplasmic microtubules in the NK cells of these drug-treated NK–TC pairs were allowed to reassemble by washing out the drugs, in essentially all such pairs the NK MTOC was now found oriented towards the cell–cell contact area. This suggests that (*i*) cytoplasmic microtubules in the NK cells are di-

rectly or indirectly involved in the reorientation of the MTOC, and (*ii*) the signal for the MTOC reorientation in the NK cell that comes from the bound target persists during the time the two cells remain in contact (i.e., is not a transient signal that rapidly decays after cell–cell contact is made). This signal is unidirectional: the MTOC of the TC did not reorient.

The microtubule assembly status is also important in the expression of cytotoxicity with this clone of NK cells. Microtubule depolymerization strongly inhibited cytotoxicity, and microtubule repolymerization after washing out the drugs allowed its nearly complete recovery. In interpreting these results, it should be realized that microtubule disassembly generally has as one of its consequences a redistribution of Golgi elements from their normal compact perinuclear configuration to a widely dispersed state near the cell periphery (14, 15).

In other unpublished experiments using the NK-resistant cell line YAC-8 (1, 16), we have found that conjugates were formed with the cloned NK cells but that, even 50 min after mixing, the orientation of the MTOC inside the NK cells of such pairs was random with respect to the cell contact area.

All of these findings are consistent with the hypothesis and the evidence (2, 3) that NK-induced cytotoxicity is the result of a stimulated secretion of toxic components from the NK cell to the TC with which it is in contact. It is proposed that the reorientation of the GA and MTOC in the NK cell that is bound to its target serves to direct the traffic of Golgi-derived secretory vesicles to the plasma membrane of the NK cell at the area of cell–cell contact. Fusion of the secretory vesicles with the plasma membrane releases the toxic components into the confined intercellular space and results in their unidirectional uptake by the apposed plasma membrane of the susceptible target (2). The integrity of the cytoplasmic microtubules in NK cells is not required for NK cell–TC binding, but it may be required to maintain the compactness and polarized orientation of the GA inside the NK cell (14, 15). In addition, the microtubules may be a component of cytoskeletal tracks along which the secretory vesicles are directed from the GA to the plasma membrane. For either or both reasons, an insufficient amount of polarized GA-derived secretion may occur from the nocodazole-treated NK cell to its bound target to induce cytolysis.

Earlier evidence supporting a role for the GA in NK-mediated cytotoxic activity was obtained by Carpén *et al.* (3), who found that the ionophoric compound monensin, a drug known to disrupt GA function, irreversibly inhibited NK cell-mediated cytotoxicity. More recently, the same authors (17) studied the intracellular orientations of the GA, using a fluorescent conjugate of wheat germ agglutinin to visualize the GA in the light microscope. The cellular system they employed was a heterogeneous population of human large granular lymphocytes that included an unknown number of specific NK cells, interacting with the NK-sensitive K562 erythroleukemia target. In cell conjugates that were observed 3 hr after mixing the lymphocyte population and K562 cells, it was shown that the GA inside the majority of the K562-bound large granular lymphocytes, presumed to be specific NK cells, was oriented towards the cell–cell contact area. This result is consistent with our findings with the cloned mouse NK cells indicating that a rapid reorientation of the GA occurs upon NK–TC cell–cell contact.

There are conflicting results published concerning a requirement for microtubule integrity in NK cell-mediated cytotoxicity, with some investigators finding such a requirement (18) and others not (3). One possible explanation is that there are different types of NK cells with different characteristics (19, 20). For instance, it is conceivable that in cases in which a particularly strong interaction occurs between a NK cell type and its target, involving an especially large and interdigitated area of cell–cell contact, a sufficient degree of secretion occurs from the NK cell to its bound target to induce lysis despite the disruption of the NK microtubules and the dispersion of the GA inside the cell. In the case of a weaker NK–TC interaction, however, the degree of directed secretion from the nocodazole-treated NK cell to its target may be too small to effect lysis of the TC.

A premise upon which the studies in this paper were based—namely, that two apparently dissimilar phenomena, NK cell-mediated cytotoxicity and directed cell migration, might exhibit in common a stimulated intracellular reorientation of the GA and MTOC in the effector cell—appears to be correct. This further suggests, however, that some common features exist in the stimulus–response mechanisms in these two cases that result in the similar GA/MTOC reorientations. Nothing is known about the molecular basis of these mechanisms at present.

Finally, mechanisms similar to those discussed above for NK cell-mediated cytotoxicity may be involved in killing by cytotoxic T lymphocytes (CTL). Using cloned lines of CTL, Dennert and Podack (21) obtained electron microscopic evidence that secretory components are transferred from the T cell to its bound target; these components closely resemble structurally the components transferred between a NK cell and its bound TC. Furthermore, Geiger *et al.* (22) have shown that within the CTL of CTL–TC conjugates, the MTOC is oriented towards the area of cell–cell contact.

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